Original Article

Impact of Immunohistochemical Expression of Collagen III and MMP-14 in Odontogenic Keratocyst

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Abstract

Objective: Immunohistochemical expression of collagen III and MMP14 was evaluated in odontogenic keratocyst, and correlated to the clinicopathological parameters to remark on the role of these markers in the biological behavior of the cyst.

Methods: Twenty-three odontogenic keratocysts samples were collected from three pathological laboratories in Sulaimani. Demographic information and the available radiographic investigation were recorded. The immune expression of collagen III and MMP14 was evaluated and related to the clinicopathological variables. Chi-square test was used for analysis and a p-value of 0.05 was cut off point for significance.

Results: Males were predominantly affected by odontogenic keratocysts lesions (69.6%), which were mainly detected in the >35 years age groups. Keratocyst lesions were seen more frequently in the mandible (82.6%), with well-defined borders, and unilocular appearance. Collagen III was oriented more commonly in a parallel direction (70.8%), with moderate intensity (45.8%). MMP14 revealed cytoplasmic expression in the epithelial lining of the keratocyst (100%), score 3 was the most prevalent expression (54.2%) in the cystic wall, and prominent inflammation and epithelial separation were detected with high scoring of MMP14, which were (57.1%) and (56.3%) respectively.

Conclusions: Both markers had independent combined roles in the cyst's biological behavior, including the prevalent parallel direction of the collagen III, while overexpression of the MMP14 might have an impact role in the aggressive behavior of the lesion.

Keywords: *Immunohistochemical, Keratocyst, MMP14, Collagen III, Sulaimani*.

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Introduction

Odontogenic keratocyst is a developmental odontogenic cyst that arises from the remnant of the dental lamina[1]. It may occur as a sporadic cystic lesion or it may be associated with nevoid basal cell carcinoma syndrome (NBCC)[2].

According to the WHO in 2005, OKC was classified as an odontogenic tumor due to its aggressiveness and high recurrence rate[3]. In 2017, WHO reclassified OKC as a cyst rather than a tumor as there was not enough evidence to support the neoplastic nature and the terminology changed to odontogenic keratocyst[4]. Still, this cyst has had special consideration over other jaw cysts due to its potential aggressiveness, high recurrence rate, and its association with NBCC. OKCs revealed male predilection and affected young adults with ages ranging from (20 -30) years[5]. This cyst was detected more commonly in the mandible, especially in the posterior area. It demonstrated a well-defined radiolucent area with smooth and often corticated margins, with 25% to 40% of cases associated with an unerupted tooth[6].

The epithelial lining is characterized by corrugated keratinized stratified squamous epithelia with a thickness of 6 to 8 cell-layer. The basal layer is composed of a columnar hyperchromatic, palisaded, polarized nucleus. The flat junction between epithelial and connective tissue walls and the absence of retre ridge of epithelia make the separation between lining and wall easier than in other cysts. In general, the wall is thin and friable with no inflammation[7].

Collagen III has thin, loosely packed fibrils that constitute 5-20% of the entire collagen content of human collagen fibers[8]. It is expressed normally in the dermis, aorta uterus and bowel and appears in the different stages of embryonic development[9]. It is produced by fibroblasts and other mesenchymal cells and plays a significant role in inflammation, with recorded expression in lung damage, viral liver disease, kidney fibrosis, and vascular disorders. [10] Several soft tissue tumors reported high expression of collagen III[11].

MMP14, also called MT1-MMP, is a member of the matrix metalloproteinase (MMP) family and a calcium-dependent, zinc-containing endopeptidase[12]. It cleaves gelatin, fibronectin, and laminin, and plays an important role in MMP2 processing and activation[13]. High expression of MMP14 is associated with a poor prognosis, invasiveness, and metastasis of cancerous cells[14]. Furthermore, high expression of MMP14 in ameloblastoma identified the role of this marker in the local invasion of the tumor[15]. In this research, the expression of MMP14 and collagen III was evaluated in OKCs, and correlated to the clinicopathological parameters to remark on the role of these markers in the biological behavior of the cyst.

Materials and methods

Study Design

Histologically diagnosed OKC 24 lesions of 23 patients were obtained from the archives of the Department of Oral Pathology-College of Dentistry at Sulaimani University, Shorsh pathological lab, and a private lab. Furthermore, three cases of dentigerous cysts and two cases of ameloblastoma were used as controls. Available clinical data were registered from archival case sheets, including demographic data (age, sex, and site) and radiographic investigations (uni or multilocular radiolucency, borders, penetration of cortex and other adjacent vital structures, root resorption, and association with an impacted tooth). The ethical committee of the college of dentistry approved the study (code no. 60/21 on 9/11/2021).

Immunohistochemical staining and evaluation

Three 5 µm thick sections were cut from each tissue block and mounted on positively charged slides.

1. They were deparaffinized in xylene and rehydrated through a series of ethanol.
2. The antigen was retrieved by boiling the sections with citrate buffer (pH-6, 15min).
3. Endogenous peroxidase activity was blocked by hydrogen peroxidase (10 min), then a protein block was applied (10 min).
4. Sections were incubated with primary antibodies (rabbit polyclonal anti-Collagen III, and rabbit polyclonal anti-MMP-14, dilution 1:100, Abcam; UK) for 45 min and then washed with PBS.
5. Sections were incubated with complement (10 min) and washed with PBS. Goat anti-rabbit HRP conjugate was added for 15 min and washed.
6. Finally, sections were stained by DAB (5 min in the dark) and counter-stained with hematoxylin (20 sec). Lastly, the slides were dehydrated, cleared, and mounted with DPX to be ready for microscopical examination.

Positive controls were used according to manufacturer protocols. While the negative control was included in each run by omitting the primary antibody and applying antibody diluents alone. Digital images were acquired

from each section of stained slides using an Am Scope AF205 digital camera. The five most representative fields were selected for image analysis using the Image J 1.44 program. The orientation of collagen was evaluated according to the epithelial lining of the cysts under x100 magnification and classified as (1) non-parallel and (2) parallel[16] and the organization of collagen near and distant from epithelia was classified as loose (1) and dense (2). Finally, the intensity was graded as weak (+), moderate (++), and strong (++++)[11].

The MMP -14 epithelial expression was evaluated in (basal, and supra-basal layers). With the intensity of (mild, moderate, and strong). While fibroblast expression of the cyst wall was scored into: 0 (≤ 10% immunostained cells), 1 (11–25% immunostained cells), 2 (26–50% immunostained cells), 3 (51–75% immunostained cells), and 4 (> 75% immunostained cells)[17].

Statistical Analysis. Data from the studied samples were entered into the Statistical Package for the Social Sciences (SPSS) version 19. The frequency of distribution of the studied variables was registered. The Chi-square test was used to analyze the relation of immunoscoring to clinicopathological parameters. The significant p-value was considered to be less than 0.05.

Results

Clinical findings

This study showed OKCs were predominant in males (69.6%). Females were slightly more prevalent in the younger age group (57.1%), while patients over 35 years were predominantly males (81.2%) (Table 1). Most cases were located in the lower jaw (82.6%) and were more frequently seen in males (65.2%). Lastly, a significant difference was detected between age grouping and sex (p= 0.05) (Table 1). Regarding site distribution, twenty-four OKCs were reported, 11 lesions (45.8 %) occurred on the right side, 8 cases (33.3%) on the left side, and two (8.3%) showed midline distribution. However, three patients (12.5%) showed missing detailed side specifications. Radiographically all are discussed in (Figure 1-2) . All the 3 cases of the dentigerous cyst had well-defined lesions around the impacted tooth (1 canine, and 2 third molars.). One case of ameloblastoma involved a lower right third molar, and the lesion had a corticated border. The other case was located in the mandibular right posterior region with thinning of the cortical bone.

Immunohistochemical findings of collagen III

The human skin was used as a positive control for this marker, the dermal connective tissue and the basement membrane of the epidermis of the skin were collagen III positives, Fig 3, A. The connective tissue wall of the dentigerous cysts was densely packed, run in a non-parallel way (n= 3, (100%), and showed mild intensity, Fig 3, B. In ameloblastoma, the collagen fibers were running in a non-parallel orientation, loosely packed, and showed strong intensity (n=2, 100%), Fig 3, C. In OKCs samples, collagen fibers orientation in the connective tissue wall was predominantly parallel (70.8%), Fig 3, D. Both organizations of collagen fibers were equally seen (50 % each), Fig 3, E, F. While the moderate intensity was predominant (45.8%), Fig 3, E, (Table 2).

Immunohistochemical findings of MMP14

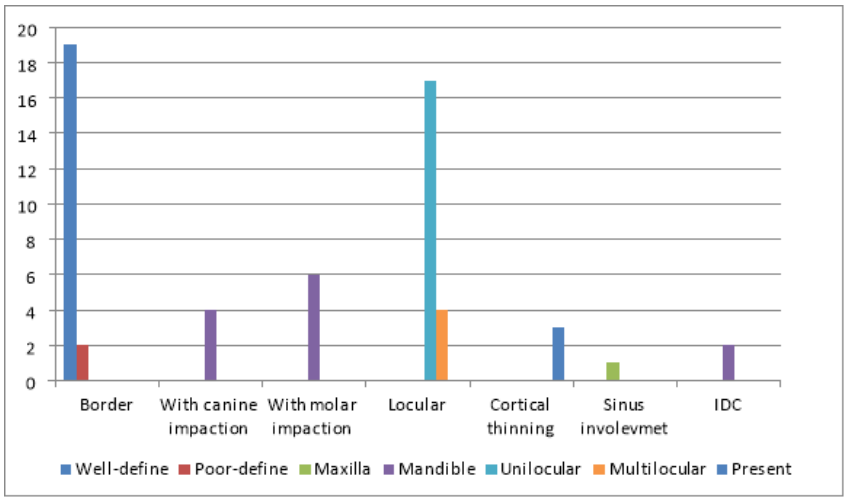
Breast carcinoma was used as a positive control and showed strong cytoplasmic expression only, Fig 4, A. In all dentigerous cyst samples in the epithelial lining, it showed mild cytoplasmic positive expression (n=, 100%), Fig 4, B. The connective tissue wall had score 2 expressions. While epithelial islands of ameloblastoma (2 cases) had strong expression in the outer cell layer and mild expression in stellate reticular cells, Fig 4, C. Furthermore, fibroblasts in the connective tissue surrounding the epithelial islands had score 3 expressions. In the OKC samples, all cystic epithelial linings had positive cytoplasmic expression (n=24, 100%), Fig 4. Positive nuclear expression was seen in the lining of 3 cases (12.5%), Fig 4, D. The intensity of basal cell layers was mild in 11 cases, moderate in 7 cases, and strong in 4 cases. Two lesions in the basal cell layer were negative, Fig 4, E. The supra-basal cell layer showed 100% positive expression (intensity was mild in 8 cases (33.3%)), Fig 4, F, moderate in 14 cases (58.3%), Fig 4, E, and strong in 2 cases (8.3%), Fig 4, D. In the connective tissue wall of all 24 cases, positive staining was detected in fibroblast, score 3 was the predominant finding: n=13 (54.2%), Fig 4, E, followed by score 2 which accounted for 8 cases (33.3%). A non–significant relation was found between immunoscoring and the clinicopathological parameters as shown in (Table 3) and (Figure 3).

Correlation between MMP14 and Collagen III in cyst wall

Statistically, the relationship between these two immunohistochemical markers revealed a non-significant relation, as the p-value was (0.207) between the direction of collagen III and expression of MMP14 in the OKC wall. While a significant relationship was detected between these two markers when collagen III intensity of collagen fibers in the connective tissue wall became stronger with increasing expression of MMP14, as the p-value was (0.05).

Table 1: The distribution of 23 OKC patients regarding sex, age, and site.

Sex	Total		Age				Site*				Total	
			≤ 35		>35		Maxilla		Mandible			
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Male	16	69.6	3	18.8	13	81.2	2	8.7	15	65.2	17	73.9
Female	7	30.4	4	57.1	3	42.9	3	13	4	17.4	7	30.4
p-value	0.05						0.06					



- IDC: inferior dental canal.

Figure 1: Impaction and findings of the radiographical evaluation of patient samples.

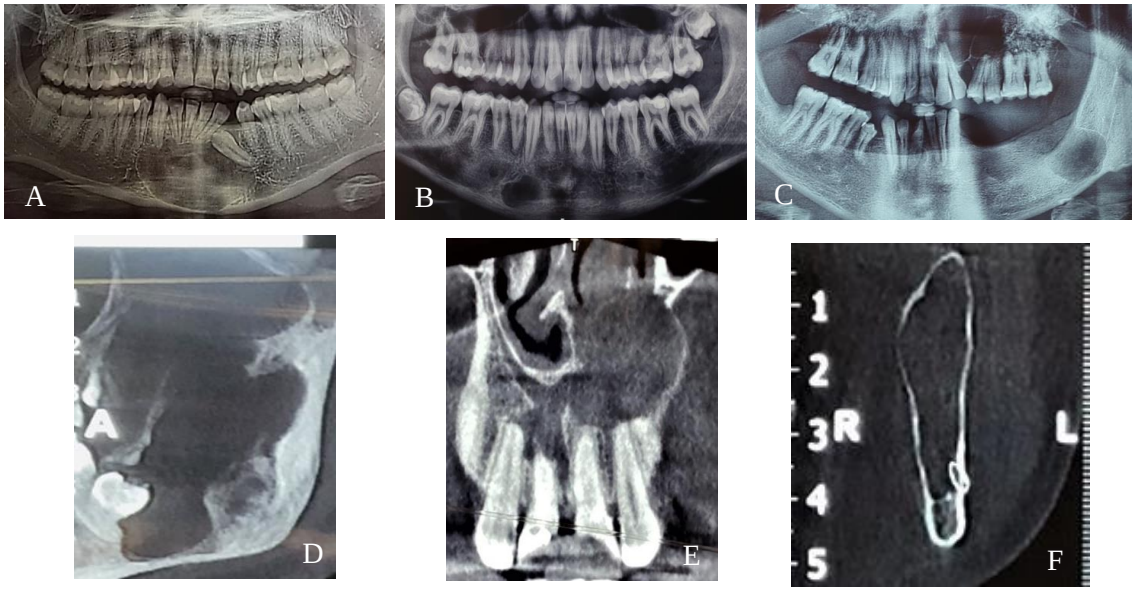


Figure 2: OPG radiographs of OKC show lesions with: A) a well-defined border in the anterior mandible associated with the impacted left canine. B) Poorly defined border in the lower right mandible. C) Multilocular, well-defined border in the upper right of the maxilla, another unilocular lesion with a well-defined border from the same case in the lower left posterior. CBCT sections show OKC lesions with involvement of D) cortical bone thinning of the mandible**.** E) Maxillary left sinus with floor resorption. E) Inferior nerve canal.

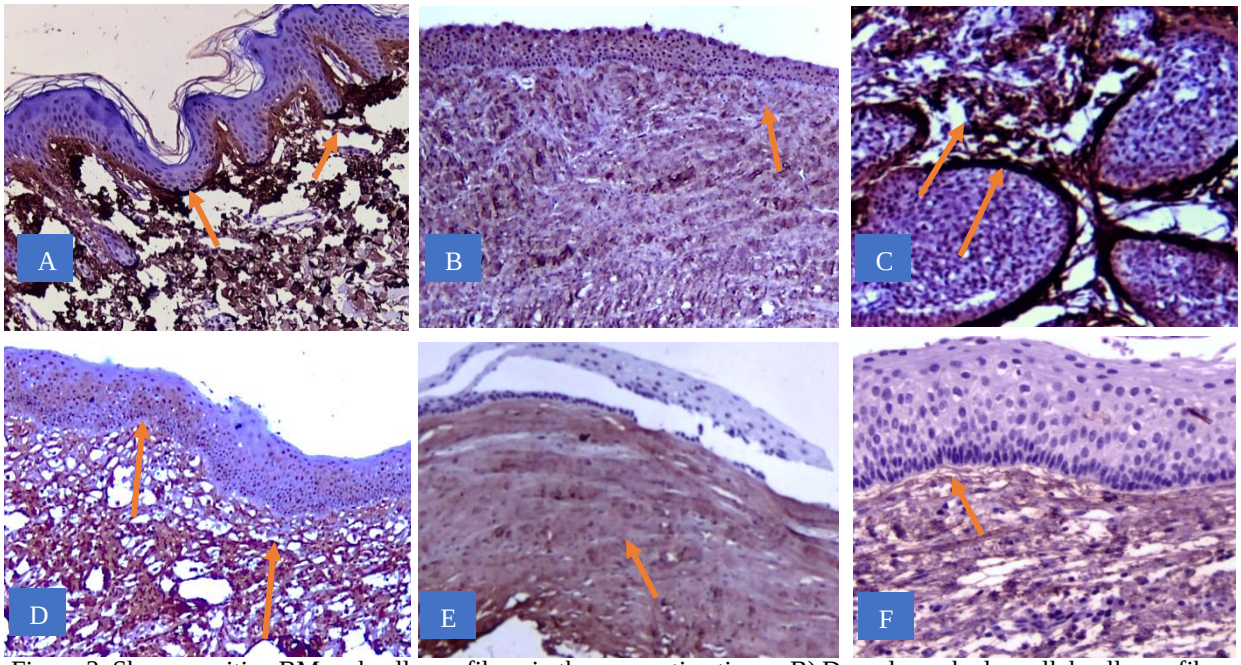


Figure 3: Shows positive BM and collagen fibers in the connective tissue. B) Densely packed parallel collagen fibers with mild intensity in the dentigerous cyst wall. C) Strong intensity, loosely packed non-parallel collagen fibers in ameloblastoma background. D) Loosely packed non-parallel collagen fibers, with moderate intensity in OKC. E) Moderate intensity of densely packed parallel collagen fibers in OKC. F) Moderate intensity of loosely packed parallel collagen fibers in OKC.

Table 2. The orientation, organization, and intensity of collagen III fibers s in OKCs lesions.

Connective tissue wall	Orientation			Organization			Intensity		
		No	%		No	%		No	%
	Parallel	17	70.8	Loose	12	50	Weak	6	25
	Non-parallel	7	29.2	Dense	12	50	Moderate	11	45.8
	Total	24	100	Total	24	100	Strong	7	29.2

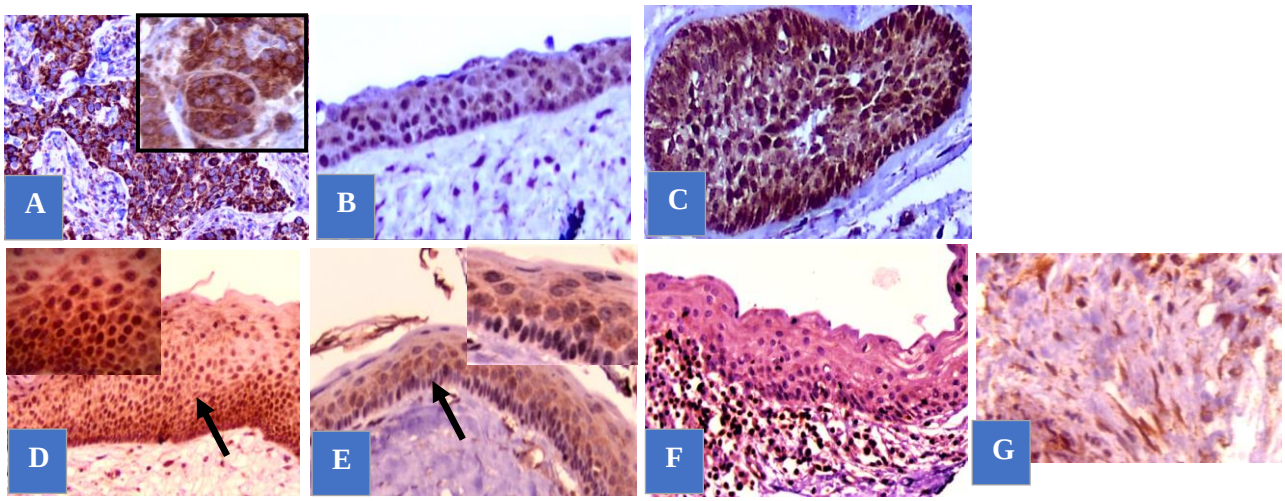


Figure 4: Microscopic sections show the expression of MMP14. A) positive strong expression of MMP14 of nucleus and cytoplasm of basal and suprabasal layers of OKC. B) Moderate cytoplasmic expression of suprabasal layers with negative basal layer (cytoplasm & nucleus) of OKC. C). Mild expression of cytoplasm in basal and suprabasal layer and negative nuclear of OKC. D) Mild cytoplasmic expression of DC epithelial lining. E) AB shows strong expression of the outer cells of the epithelial island and mild expression of stellate cells. F) Positive fibroblastic expression of the connective tissue wall of OKC (10X).

Table 3: MMP-14 immunoscoring in connective tissue wall concerning clinicopathological information.

Wall		Score 1		Score2		Score3		P value
		No	%	No	%	No	%	
Connective tissue scoring		3	12.5	8	33.3	13	54.2	
Sex	Male	2	6.3	6	53.8	9	56.3	0.947
	Female	1	0	2	42.9	4	57.1	
Age	≤35	0	0	4	57.1	3	42.9	0.397
	>35	3	6.3	6	37.5	10	62.5	
Presence of inflammation	yes	3	5.3	7	36.8	11	57.9	0.306
	No	0	0	3	60	2	40	
Epithelial separation	yes	2	6.3	6	37.5	9	56.3	0.446
	No	1	0	4	50	4	50	
Border	Well-define	2	66.7	5	62.5	12	92.3	0.220
	Poor-define	1	33.3	1	12.5	0	0	
Sinus involvement	yes	0	0	1	20	0	0	0.250
	No	5	100	4	80	0	0	
IDC	Yes	0	0	0	0	3	23.1	
	No	3	100	8	100	10	76.9	

Discussion

The high recurrence rate of keratocysts has drawn special attention to understanding the epithelial lining and the stromal wall pathological changes. Keeping in mind that there has been controversy over the classification of OKC, as it was identified as a tumor in 2005 WHO classification[3], then it was returned to a cyst in 2017[4]. In Sulaimaniah, from 2008-2019, OKC prevalence was 22.3% of all odontogenic cysts, which was higher than the prevalence ranging from 14%-16% of all odontogenic cysts that was reported in two other Iraqi studies[18,19]. However, OKC prevalence among the odontogenic cysts was very low in a Turkish study (4.8%)[20].

In this study, males were affected nearly three times more than females (2.28:1). This was similar to the Mohanty et al. finding in a 20-year experience study (2.5:1)[21]. Furthermore, another study also indicated male predominance (2.8:1)[20]. On the other hand, a previous comparative study showed slight female predominance. This difference may be due to the selected sample size[22].

With age, the patient has a high chance of being diagnosed with OKC, because this lesion mostly is symptomless, and causes no swelling due to its anterioposterior expansion. It could be diagnosed in the routine radiograph or during searching for unerupted or wisdom teeth, or in the case of a symptomatic OKC inflamed cyst[23]. This concept was in line with the current study where the frequently affected age group was patients over 35 years old and the mean age was (38±15). Karaca et al. reported the mean age of OKC patients to be 45 years[24]. A 12-year follow-up study showed a predominance of OKC lesions in the third to fourth decade of life[23].

A previous study reported that OKC in the mandible was more predominant than in the maxilla, especially the posterior region[25], which supports the finding of this study.

A study conducted in 2018 revealed that lesions associated with impaction, including dentigerous cyst, OKC, and ameloblastoma in the mandible, had different prevalences of (46.6%), (29.8%), and (9.2%), respectively, and most of the impaction located in the mandible[26]. In this study (41.7%) of OKCs were associated with the impacted tooth, and were located in the mandible and (79.2%) of all the lesions had a well-defined border. This was very similar to the Kitisubkanchana et al. findings, as (47%) of their lesions were associated with the impacted tooth, and (60%) of the cysts had a well-demarcated border[27].

Sánchez et al. found that unilocular lesions (71%) were more predominant than multilocular lesions (29%)[28], which supports our findings. Furthermore, in this study (14.3%) of OKC showed perforation and thinning of the cortical plate. While in another study, only 1.6% of cases had cortical thinning[21]. However, a conclusion could not be obtained as limited radiographical images were collected in this research from the patients.

Since the predominant component of the stroma is collagen, studying collagen fibers may be useful in understanding cystic and tumor behavior, as the biological activity of these lesions does not depend on the presence of epithelium alone but also on the

supporting stroma. Thin, loosely packed collagen III fibrils were mostly found in inflammatory cases and tumors[29].

In this research, collagen III revealed expression in all OKCs cystic wall, which supports the finding of other studies[30,31] and might indicate the tumor behavior of this cyst.

In the present study, collagen fibers were running predominantly in the parallel direction in the OKC wall, while a non-parallel pattern was prevalent in both dentigerous cysts and ameloblastoma. Similar findings were seen in the Harkanwal et al. and Sabrina P et al. studies, as they reported more predominant parallel collagen fibers in OKC lesions, while non-parallel orientation was detected in the dentigerous cyst and radicular cysts. They explained this difference as an important factor in facilitating the separation of the epithelial lining from the underlying connective tissue wall and promoting ECM degradation and infiltrative growth[16,33]. The current study reported that the intensity of collagen III fibers was significantly increased with the remarked presence of inflammatory cells in the cystic wall of OKCs as P value was (0.004). One may speculate that inflammatory mediators could have a role in the fragmentation of cystic walls and increased recurrence chance later on.

The current study showed that MMP14 had cytoplasmic expression in all OKC cases at the epithelia lining and connective tissue wall. This finding was in line with the Amm et al. results, as they reported cytoplasmic expression for MMP14 in all OKCs samples. However, they did not determine the epithelial layers localization specifically because they studied only four cases[34]. Ribeiro et al. showed higher expression of MMP14 in the basal cell layer than in the supra-basal layers[35], which conflicts with the finding of this study.

The high scoring of MMP14 in the connective tissue wall of OKC supports the significant role of this marker in degrading extracellular matrix proteins, which further facilitates the invasion capacity of this lesion[36]. In this study, the epithelial lining separation and presence of inflammation were increased with high scoring of MMP14 in the cyst wall, which could give the impact role of overexpression of this marker in degrading basement membrane with the unneglectable role of inflammation in matrix degradation of the cystic wall and aggressive behavior of cyst[37].

In ameloblastoma, MMP14 showed a high expression, which was similar to previous studies[38-40]. Dentigerous cysts showed less scoring of MMP14 in comparison to OKCs lesions in the present study. This finding revealed that OKC behaves more like a tumor than a cystic lesion. Unfortunately, no previously published English literature studied MMP14 expression in dentigerous cysts.

Conclusion

The predominant parallel orientation of collagen III might affect the behavior of cysts with no impact role of the organization of these fibers. On the other hand, an increased score of MMP14 was markedly associated with epithelial separation and matrix degradation with the invasive ability of the lesions. Lastly, larger samples with other MMP14 enzymes of the cyst wall should be studied.

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